

# REPORT DOCUMENTATION PAGE

Form Approved

OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 9/14/94	3. REPORT TYPE AND DATES COVERED <i>Final 15 Jul 91 - 14 Jul 94</i>	
4. TITLE AND SUBTITLE Enzyme Design for Nonaqueous Media: Optimization of Enzymatic Catalysis for Organic Solvent Systems.			5. FUNDING NUMBERS  <i>DAAL03-91-G-0224</i>	
6. AUTHOR(S)  Jonathan S. Dordick and Douglas S. Clark				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Iowa Department of Chemical and Biochemical Engineering Contact: Jonathan S. Dordick 129 CB, Iowa City, IA 52242			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Research Office P. O. Box 12211 Research Triangle Park, NC 27709-2211			10. SPONSORING/MONITORING AGENCY REPORT NUMBER  <i>ARO 28699.7-L5</i>	
11. SUPPLEMENTARY NOTES The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.				
12a. DISTRIBUTION/AVAILABILITY STATEMENT  Approved for public release; distribution unlimited.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) From abiotechnological perspective there are many potential advantages of employing enzymes in organic as opposed to aqueous media. To that end, we have concluded an initial three-year research program in the area of enzymatic catalysis in organic solvents. Our studies have focused on the effect of protein hydration on subtilisin BPN' and Carlsberg in nonaqueous media. Investigations on protein engineered mutants, catalyst engineering studies, and structural studies, primarily employing EPR spectroscopy, have revealed fundamental information on the role of water, the the nature of enzyme structure, and the effects of solvents on the catalytic activity in organic solvents. This study has also resulted in several rational methods to dramatically improve enzyme activity under anhydrous contitions. Catalytic activities in organic solvents can now be expressed at levels similar to that in water--this is a major advance in the field of nonaqueous enzymology.				
14. SUBJECT TERMS  Enzymes in organic solvents, subtilisin catalysis, Activation of catalysis, Dehydrated media.			15. NUMBER OF PAGES 4	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UL	

DTIC QUALITY INSPECTED 4

19950203 339

## GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

**Block 1. Agency Use Only** (*Leave blank*).

**Block 2. Report Date.** Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

**Block 3. Type of Report and Dates Covered.** State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

**Block 4. Title and Subtitle.** A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

**Block 5. Funding Numbers.** To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

<b>C</b> - Contract	<b>PR</b> - Project
<b>G</b> - Grant	<b>TA</b> - Task
<b>PE</b> - Program Element	<b>WU</b> - Work Unit Accession No.

**Block 6. Author(s).** Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

**Block 7. Performing Organization Name(s) and Address(es).** Self-explanatory.

**Block 8. Performing Organization Report Number.** Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

**Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es).** Self-explanatory.

**Block 10. Sponsoring/Monitoring Agency Report Number.** (*If known*)

**Block 11. Supplementary Notes.** Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

**Block 12a. Distribution/Availability Statement.** Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

**DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."

**DOE** - See authorities.

**NASA** - See Handbook NHB 2200.2.

**NTIS** - Leave blank.

**Block 12b. Distribution Code.**

**DOD** - Leave blank.

**DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.

**NASA** - Leave blank.

**NTIS** - Leave blank.

**Block 13. Abstract.** Include a brief (*Maximum 200 words*) factual summary of the most significant information contained in the report.

**Block 14. Subject Terms.** Keywords or phrases identifying major subjects in the report.

**Block 15. Number of Pages.** Enter the total number of pages.

**Block 16. Price Code.** Enter appropriate price code (*NTIS only*).

**Blocks 17. - 19. Security Classifications.** Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

**Block 20. Limitation of Abstract.** This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

## BRIEF OUTLINE OF RESEARCH FINDINGS

From a biotechnological perspective there are many potential advantages of employing enzymes in organic as opposed to aqueous media. To that end, we have concluded an initial three-year research program in the area of enzymatic catalysis in organic solvents. Our studies have focused on the effect of protein hydration on subtilisins BPN' and Carlsberg in nonaqueous media. Investigations on protein engineered mutants, catalyst engineering studies, and structural studies, primarily employing EPR spectroscopy, have revealed fundamental information on the role of water, the nature of enzyme structure, and the effects of solvents on the catalytic activity in organic solvents. Our findings can be summarized as follows:

- \* Protein engineering has been shown to result in catalysts with activities over 100-fold higher than for the wild-type subtilisin BPN' in dry hexane (as a representative organic solvent).
- \* Dehydration of subtilisin appears to affect the active-site region and destabilize the charged transition-state of the enzyme-catalyzed transesterification reaction. This lost free energy of stabilization may be partially regained by increasing the hydration of the enzyme's active site.
- \* Addition of 5  $\mu\text{L/mL}$  of water to a THF solution results in a 10-fold increase in the catalytic efficiency of BPN'.
- \* The local polarity (as measured through EPR spectroscopic studies) increases sharply as water is added up to ca. 20  $\mu\text{L/mL}$ , then levels off at higher water concentrations.
- \* An independent method to increase the active-site polarity is to modify the active-site structure by site-directed mutagenesis. Gly<sub>166</sub>→Asn (G166N) and Met<sub>222</sub>→Gln (M222Q) mutations were used and showed increased activity relative to the wild-type in THF as compared to water.
- \* Immobilization of chymotrypsin onto porous glass beads improves catalysis from 17- to 67- fold, depending on the solvent.
- \* Simple non-buffer salts can dramatically activate enzyme function in organic solvents. Subtilisin Carlsberg freeze dried into a powder which contained 98% (w/w) KCl, 1% (w/w) enzyme, and 1% (w/w) phosphate buffer was nearly 3,750-fold more active in hexane ( $k_{\text{cat}}/K_{\text{m}} = 390 \text{ M}^{-1}\text{s}^{-1}$ ) than enzyme prepared in the absence of KCl (whose  $k_{\text{cat}}/K_{\text{m}}$  is similar to that obtained upon freeze drying the enzyme from 20 mM phosphate buffer).
- \* A simple approach to the solubilization of enzymes in organic solvents has resulted in catalytic efficiencies similar to that in water. Thus, for subtilisin Carlsberg, values of  $k_{\text{cat}}/K_{\text{m}} > 4,000 \text{ M}^{-1}\text{s}^{-1}$ .

These last two points indicate that enzymatic catalysis in organic solvents can be as efficient as in water. This is critical for the further understanding and use of enzymes in dehydrated environments.

Much of this work is now published or in the process of being published as shown on the following page.

## Publications

1. R. Affleck, Z.-F. Xu, V. Suzawa, K. Focht, D. S. Clark, and J. S. Dordick (1992), "Enzymatic Catalysis and Dynamics in Low-Water Environments", Proc. Natl. Acad. Sci. USA **89**, 1100-1104.
2. R. Affleck, C. A. Haynes, and D. S. Clark (1992), "Solvent Dielectric Effects on Protein Dynamics", Proc. Natl. Acad. Sci. USA **89**, 5167-5170.
3. Z.-F. Xu, K. Focht, and J. S. Dordick (1992), "Engineering Subtilisin for Use in Organic Solvents", Ann. N. Y. Acad. Sci. **672**, 94-99.
4. J. Kim and J. S. Dordick (1993), "Pressure Affects Enzyme Function in Organic Media", Biotechnol. Bioeng. **42**, 772-776.
5. Z.-F. Xu, R. Affleck, P. Wangikar, V. Suzawa, J. S. Dordick, and D. S. Clark (1993), "Transition State Stabilization of Subtilisins in Organic Media", Biotechnol. Bioeng. **43**, 515-520.
6. P. P. Wangikar, T. P., Graycar, D. A. Estell, D. S. Clark, and J. S. Dordick (1993), "Protein and Solvent Engineering of Subtilisin BPN' in Nearly Anhydrous Organic Media", J. Am. Chem. Soc. **115**, 12231-12237.
7. Yu. L. Khmelnitsky, S. H. Welch, D. S. Clark, and J. S. Dordick (1994), "Salts Dramatically Enhance Activity of Enzymes Suspended in Organic Solvents", J. Am. Chem. Soc. **116**, 2647-2648.
8. V. M. Paradkar and J. S. Dordick (1994), "Aqueous-Like Activity of  $\alpha$ -Chymotrypsin Dissolved in Nearly Anhydrous Organic Solvents", J. Am. Chem. Soc. **116**, 5009-5010.
9. A. M. Blinkovsky, Yu. L. Khmelnitsky, and J. S. Dordick (1994), "Organosoluble Enzyme-Polymer Complexes: A Novel Type of Biocatalyst for Nonaqueous Media", Biotechnol. Techniques **8**, 33-38.
10. P. P. Wangikar, D. Carmichael, D. S. Clark, and J. S. Dordick (1994), "Active-Site Titration of Enzymes in Organic Solvents", Biotechnol. Bioeng. (submitted).
11. P. P. Wangikar and J. S. Dordick (1994), "Probing Enzyme Transition State Hydrophobicities", Biochemistry (submitted).
12. V. Suzawa, Y. L. Khmelnitsky, J. S. Dordick, and D. S. Clark (1994), "Conformational Studies of Partially Hydrated Enzyme Immobilized and Suspended in Organic Solvents", (in preparation)
13. V. Suzawa, Y. L. Khmelnitsky, J. S. Dordick, and D. S. Clark (1994), "Structural and Dynamic Properties of Activated Enzyme-Salt Catalysts in Organic Media" (in preparation).
14. P. P. Wangikar, P. A. Michels, D. S. Clark, and J. S. Dordick (1994), "Structure, Function, and Dynamics of Enzymes Dissolved in Organic Solvents", (in preparation).

## Patent Application

J. S. Dordick, D. S. Clark, and Y. L. Khmelnitsky (1994). "Compound and Process to Enhance Activity of Enzymes Suspended in Organic Solvents", U. S. Patent Pending.

## Students and Postdocs Supported

1. Rhett Affleck - Ph.D. U. C. Berkeley
2. Valerie Suzawa - Ph.D. Candidate, U. C. Berkeley
3. Peter Michels - Ph.D. Candidate, U. C. Berkeley
4. Pramod Wangikar - Ph.D. Candidate, U. Iowa
5. Jungbae Kim - Ph.D. Candidate, U. Iowa
6. Yuri Khmelnitsky - Postdoc, U. Iowa

Accession For	
NTIS CRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification _____	
By _____	
Distribution /	
Availability Codes	
Dist	Avail and/or Special
A-1	